J. Plant Biotechnology (2006) Vol. 8 (1), pp. 37~41

Journal of Plant Biotechnology

In vitro Multiple Shoot Proliferation and Plant Regeneration of *Vanilla planifolia* Andr. - A Commercial Spicy Orchid

C. Gopi[1*], T.M. Vatsala[2], P. Ponmurugan[1]

[1]Department of Biotechnology, School of Biotechnology, K.S.Rangasamy College of Technology, Tiruchengode-637209, Tamilnadu, India; [2]T.M.Vatsala-Shri AMM Murugappa Chettiar Research Centre, Tharamani, Chennai-600 113, Tamilnadu, India

Abstract

***In vitro* mass multiplication of *Vanilla planifolia* was investigated using node as explant. Multiple shoots were developed in MS medium supplemented with 2.0 mg l^{-1} 6-benzylaminopurine and 1.0 mg l^{-1} α-naphthalene acetic acid. Multiple shoots were maintained for 6-7 weeks with regular subculturing at the end of 3rd week onto fresh medium. The maximum number of shoots at the rate of 12.8 per node segment was achieved over a period of four weeks. The elongated shoots were separated from the shoot clusters and were transferred onto half strength MS medium supplemented with indole-3-acetic acid (1.0 mg l^{-1}) over a period of 28 days for induction of roots. The development of roots was observed on 7th day of incubation. The *in vitro* raised plantlets were transferred to poly-cups, covered with polyethylene sheets and maintained under shade net for 25 days for hardening. Finally these plants were transferred to field and recorded that 85 % of tissue cultured plants were survived. From the present study, a simple and efficient micropropagation protocol was developed for *Vanilla planifolia* using single node segments as explants.**

Key words: *Vanilla planifolia*, *in vitro* propagation, multiplication, node segments, and plantlet

Introduction

Vanilla (*Vanilla planifolia* Andr. Orchidaceae) is an important spice crop and offers excellent scope for cultivation in the tropical high rainfall regions in Southern India. There is a growing demand for natural *Vanilla* flavour in the global trade (Rao and Ravishankar 2000). *Vanilla* essence, extracted from the cured beans is widely used for flavoring cakes, sweets, chocolates, ice creams, beverages, cosmetics and perfumery industries including condiments and oleoresins trade (Rao and Ravishankar 2000; Giridhar et al. 2001; Geetha and Shetty 2000).

Vanilla is generally grown as an intercrop in plantations such as coffee, cardamom, coconut and arecanut, in Southern India (Rao et al.1993). The plant is conventionally propagated by the stem cuttings. However, this method of propagation is rather slow, labour-intensive and time consuming (Ayyappaan1990). Tissue culture method for the regeneration of orchid is an alternative source for large-scale production of planting materials with a short span of time. *In vitro* seed germination has been attempted and culture system like regenerative callus, somatic embryo-genesis have been standardised (Nair and Ravindhran 1994). Normally seed germination is extremely difficult in nature and become an important source of variation and also scientific information on *in vitro* seed culture of Vanilla is very limited (Knudson 1946; Philip and Nainar 1986). A number of protocols have been reported for propagation of *Vanilla by in vitro* methods (Rao et al. 1993; Giridhar et al. 2001; Geetha and Shetty 2000). However, the mass propagation for commercial cultivation requires a simple, economical, rapidly multiplying and highly reproducible protocol without an intervening callus or protocorn phase so as to give raise true-to-type clones. Therefore the present investigation gives a simple and efficient protocol for *in vitro* mass production of *V. planifolia*.

* Corresponding author, E-mail: gopidurai@yahoo.com
Received Nov. 23, 2005; Accepted Mar. 20, 2006

Materials and Methods

Establishment of aseptic culture

Node segments excised from three years-old healthy vines of *Vanilla planifolia* were used as experimental material for the present study. The plants were grown in a natural partially shaded place in Shri AMM Murugappa Chettiar Research Centre, Tharamani, Chennai, Tamil Nadu. The mother plants were obtained from M/s. Parry Agro Industries Ltd., Candura-Sheikalmudi Estate, Valparai, Coimbatore District, Tamil Nadu. These explants were soaked in liquid detergent (Tween 20) for 5 min and rinsed in running tap water for 10 min. The cleaned explants were surface sterilized with 0.1% (w/v) mercuric chloride for eight minutes followed by a series of rinsing (10 times) with sterile distilled water at two min interval. The surface sterilized explants were trimmed carefully and inoculated onto Murashige and Skoog (1962) (MS) medium and gelled with 0.9% (w/v) agar. The pH of the medium was adjusted to 5.6±0.2 prior to the addition of agar. The inoculated cultures were incubated at 1500- 2000 lux under cool, white fluorescent light with a photoperiod of 16/8 hrs at 25±2°C with 70±5% relative humidity. Each treatment with ten replicates was maintained.

Multiple shoot initiation

For initiation of multiple shoots, MS medium supplemented with different concentrations of BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) were used. In addition, BAP combined with NAA in different combinations (0.25, 0.5, 1.0 and 2.0 mg l^{-1}) was also tried. Media supplemented with BAP at 2.0 mg l^{-1} and NAA at 1.0 mg l^{-1} was used for multiple shoot proliferation. These multiple shoots were trimmed about 1.5 cm and subcultured in the same medium at a periodical interval. The number of shoots developed on each nodal cutting was recorded and analyzed statistically.

In vitro rooting and hardening

In vitro established multiple shoots were carefully separated from the cluster and transferred to the half strength MS medium individually supplemented with NAA and IAA at different concentrations, (0.25, 0.5, 1.0 and 2.0 mg l^{-1}) for induction of rooting. The number of roots developed per shoot was recorded and analyzed statistically. Plantlets were removed from the medium and washed in running tap water without disturbing the root system and subsequently planted in poly-cups containing sand, vermicompost and farm yard manure mixture (1:1:1 ratio) for 20 days. The cups were covered with polyethylene sheets and maintained at 20-25°C temperature with 100 % relative humidity under shade condition. These plants were hardened for 35 days and transferred to the field.

Results and Discussion

Excised nodal explants of *V.planifolia* were cultured on MS medium with various concentrations of BAP alone and in combination with NAA for multiple shoot formation. Preliminary experiments with *V.planifolia* exhibited more contamination while using of mature explant. Whereas, young explant such as 3rd to 6th nodes showed limited contamination and this is coincide with results reported by Ganesh et al. (1996) and George and Ravishankar (1997).

The axillary buds initiated on the medium from 3rd to 4th week (Fig 1a) and elongation of the bud began from 5th week onwards. A remarkable growth was observed in the medium amended with growth regulators when compare with control. Initiation of multiple shoots was more (4.27/explant) at 2.0 mgl^{-1} than other concentration used (Table 1, Fig. 1,a-b). Further, BAP at 2.0 mgl^{-1} combined with different concentration of NAA (0.25- 2.0 mgl^{-1}) were tried (Table-1) to study the synergistic effect of auxins and cytokinin combination for the proliferation of multiple shoots and root initiation. In previous report stated that MS medium supplemented with BA and NAA combination is essential for shoot proliferation from axillary buds for Vanilla micropropagtion (Cervera, and Madrigal 1981 Kononwicz and Janick 1984; Mathew et al. 1997; George and Ravishankar 1997). According to Geetha et al. (2000), cytokinin combination with NAA is important for multiplication of Vanilla plant. In our study the observations also were made on multiple shoot proliferation and result was recorded at the concentration of 2.0 mg l^{-1} of BAP and 1.0 mg l^{-1} of NAA with average of 12.2 shoots/explant in four weeks (Fig. 1, c). George and Ravishankar (1997) reported only 5.7 shoots in same MS medium with 2.0 mg l^{-1} of BAP and 1.0 mg l^{-1} of NAA after 90 day of culture. In our study about two-fold multiplication rate was achieved when compare to BAP alone amended medium. The average shoot length was also found to be higher when compared to other concentration tested. It was therefore concluded that, 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA was necessary to obtain an optimum response in terms of more multiple shoots yield per explant. Though Geetha and Shetty(2000) have used two different media to yield about nine shoots per explant from axillary buds and 42 shoots were achieved over the period of nineteen weeks by repeated subculturing. In our studies,the effect of subculturing trimmed about 1.5 cm of multi-shoot (2-3 shoots) in the same medium resulted in proliferation of shoots was more than two-fold ratio at every subculture cycle for four week intervals

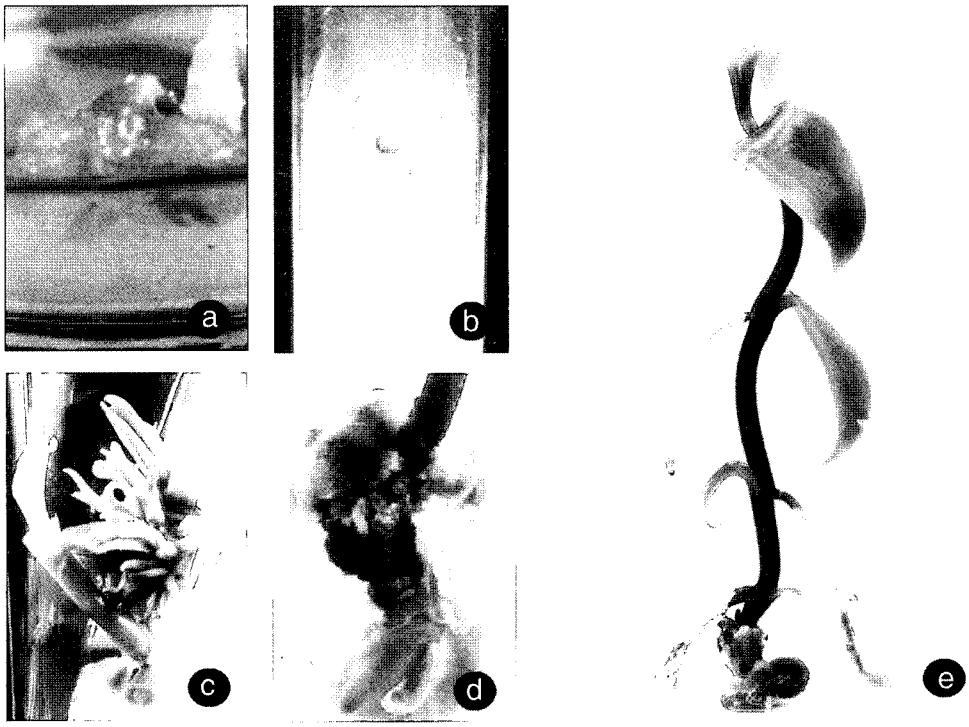

Figure 1. Various stages of *in vitro* multiple shoot proliferation and plant regeneration of *Vanilla planifolia* Andr. (a-b) Multiple shoots on MS medium containing 2.0 mg l^{-1} BAP alone. (c) Multiple shoots on MS medium containing 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA. (d) Multiple root developments from shoot on MS medium containing 1.0 mg l^{-1} IAA. (e) A well rooted plant.

Table 1. Effect of different plant growth regulators on multiple shoot formation of *V. planifolia*.

Concentration of PGRs (mg l^{-1}) in MS medium	Number of shoots/explant	Length of shoots (cm)
Hormone free medium (control)	1.0	1.5
BAP (0.5)	1.65±0.16	1.5
BAP (1.0)	2.30±0.23	1.8
BAP (2.0)	4.27±0.26	2.0
BAP (4.0)	4.12±0.26	2.2
BAP (2.0) + NAA (0.25)	6.20±0.35	2.0
BAP (2.0) + NAA (0.5)	10.3±1.56	2.2
BAP (2.0) + NAA (1.0)	12.8±0.96	2.6
BAP (2.0) + NAA (2.0)	12.2±0.83	2.5

The average of 10 replicates.
The number of shoots recorded after 4 weeks of culture.

(Table 2). Using this method, with maximum average of 120.47 shoots per explant was achieved over a period of112 days. Observation also was made on prolonged culture of multiplying clusters in MS medium resulted in distortion of shoot with stunted growth, along-with browning of shoot tips.

Shoots about three cm long were excised and cultured separately on fresh half strength MS medium individually supplemented with NAA and IAA at different concentration (0.25-2.0 mg l^{-1}) to induce rooting. The results showed that multiple rooting was observed IAA at 1.0 mg l^{-1} for a period of four weeks among other concentration tested (Fig 1, d). Percentage of rooting response of shoots was 90 with 3.50

Table 2. Effect of subculture of multiple shoots of *V.planifolia* in MS medium amended with BAP 2.0 mg l^{-1} and NAA 1.0 mg l^{-1}.

Subculture	Average number of shoots/explant*	Average height of shoots (cm)*
End of I st subculture	25.6	2.6
End of II nd subculture	58.3	2.6
End of III rd subculture	120.47	2.5
SE ±	10.08	0.04
CD at P=0.05	18.54	0.18

The average of 10 replicates.
The results recorded after 4 weeks in culture.

Table 3. Effect of different concentration of auxins on root formation of the *in vitro* grown shoots on half strength MS medium.

Types of PGRs	Different conc. of auxin (mgl^{-1})	% of shoots rooting	No. of roots/shoot	Av. length of the root (cm)
NAA	0.25	40	1.63±0.18	3.70±0.19
	0.50	55	1.80±0.24	3.85±0.41
	1.00	75	2.40±0.28	5.12±0.33
	2.00	60	1.70±0.2	3.75±0.15
IAA	0.25	55	1.65±0.16	4.00±0.18
	0.50	65	2.10±0.21	5.45±0.23
	1.00	90	3.50±0.32	6.00±0.25
	2.00	85	2.70±0.23	5.50±0.28

Data (mean ± S.E.) were recorded after 6 - 8 weeks of culture.
The average of 10 replicates and data recorded after 4 weeks of culture.

roots at the base at average length of six cm in IAA amended medium (Table 3). As the reports of Philip and Pa dikkala (1989) root induction was higher in IAA supplemented MS medium for *V. planifolia*. In earlier studies carried out by Giridhar et al. (2001) $AgNO_3$ were also used to induce the root and growth of plantlet. Whereas in our studies IAA alone was used for more roots formation for the period of three weeks. This is the new finding of formation of more than few roots at base of tissue cultured vanilla plant, as will have good survive rate during acclimatization. The well rooted plantlets harvested from the medium (Fig. 1, e) and washed thoroughly with running tap water for hardening. The results indicated that a survival rate of 85 % was achieved during the hardening of the plants. Hence the present study is of importance in developing rapid method of multiple shoot proliferation along with root development and complete plant development.

Acknowledgements

Authors are thankful to the Management of Shri AMM Murugappa Chettiar Research Centre, Tharamani, Chennai 600 113 (TN) India, for providing a research fellowship and financial assistance.

References

Ayyappan P (1990) *Vanilla* - a money making venture for ambitious farmers. Kisan World 7: 24-26

Cervera E, Madrigal R (1981) *In vitro* propagation of *Vanilla planifolia*. Environ Exp Bot 21: 441

Ganesh SD, Sreenath HL, Jayashree G (1996) Micropropagation of *Vanilla* through node culture. J Plantn Crops 24: 16-22

Geetha S, Shetty SA (2000) *In vitro* propagation of *Vanilla planifolia,* a tropical orchid. Curr Sci 79: 886-889

George PS, Ravishankar GA (1997) *In vitro* multiplication of *Vanilla planifolia* using axillary bud explants. Plant Cell Rep 6:490-494

Giridhar P, Reddy OB, Ravishankar GA (2001) Silver nitrate influences *in vitro* shoot multiplication and root formation in *Vanilla planifolia* Andr. Curr Sci 81: 1166-1170

Kononowicz H, Janick J (1984) *In vitro* propagation of *Vanilla planifolia*. HortSci 19: 58-59

Knudson L (1946) A new nutrient solution for the germination of orchid seed. Am Orchid Soc Bull 15: 214-217

Mathew MK, Rao YS, Kumar PK, Madhusoodanan KJ, Potty SN (1997) Multiplication process of *Vanilla*. Plant tissue culture and technology: Emerging Trends 67: 171-178

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol

Plant 15: 473-497

Philip VJ, Nainar SAZ (1986) Clonal propagation of *V.planifolia* (Salisb) Amesusing tissue culture. J Plant Physiol 122: 211-215

Philip VJ, Padikkala J (1989) The role of indole acetic acid in the conversion of root meristem to shoot meristem in *Vanilla planifolia.* J Plant Physiol 135: 233-236

Rao SR, Ravishankar GA (2000). *Vanilla* cultivation. J Sci Food Agric 80: 1-16

Rao YS, Mathew MK, Madhusoodanan KJ, Naidu R (1993) Multiple shoot regeneration in *Vanilla* (*Vanilla planifolia).* J Plantn Crops 21: 351-354

Nair RR, Ravindhran PN (1994) Somatic association of chromosomes and other abnormalities in *Vanilla planifolia* (Andrews). Caryologia 47: 65-73